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Improved Detection of Respiratory Syncytial Virus in Nasal Aspirates by Seminested RT-PCR

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A seminested RT-PCR for amplification of Respiratory syncytial virus (RSV)-RNA in nasal aspirates has been developed and used to test nasopharyngeal aspirates (NPAs) from 132 infants hospitalized with acute respiratory tract infections during winter epidemics. The results were compared with those obtained by virus isolation in tissue culture and antigen detection with an enzyme-linked immunosorbent assay (Ag-ELISA). RSV-RNA was detected by seminested RT-PCR in 57 of the 59 samples that were positive by virus isolation and/or ELISA, as well as in 25 of 73 samples negative by virus isolation and ELISA. Eighteen of these 25 samples were obtained from children older than one year of age, 17 of whom were experiencing reinfection, as indicated by the presence of preexisting serum RSV-IgG antibodies. These results indicate that seminested RT-PCR is more sensitive than conventional methods for the detection of RSV in patients experiencing reinfections and suggest that this assay might also be useful for rapid diagnosis of RSV infections in older people. ***J. Med. Virol. 53:366–371, 1997.***

KEY WORDS: RSV; RT-PCR; diagnosis

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most important respiratory pathogens, causing severe infections of the lower respiratory tract in infants, small children, the elderly, and in immunosuppressed patients, often requiring hospitalization [Kim et al., 1973; Whimbey et al., 1995; Agius et al., 1990; Falsey et al., 1992; Fleming et al., 1993]. Previous infection does not confer immunity and reinfections are very common [McIntosh et al., 1993; Henderson et al., 1979].The virus is highly contagious, causing yearly outbreaks of respiratory tract infections and spreads very rapidly, especially in hospital wards and nursing homes [Falsey et al., 1990, 1995]. Therefore methods for rapid and sensitive detection of RSV are required to allow the appropriate care and treatment of the patients and to reduce the risk of nosocomial infections.

In children, viral antigen detection in nasopharyngeal aspirates (NPAs) by immunofluorescence technique (IF) or by enzyme-linked immunosorbent assay (ELISA) is commonly used for the early and rapid diagnosis of RSV infections [Popow-Kraupp et al., 1986]. Although both methods are quite sensitive, negative results are obtained with a significant number of specimens in spite of clinical and epidemiological evidence for RSV infection. In addition, RSV antigen detection by IF or ELISA has not been very successful for the diagnosis of RSV infections in older children and in elderly people [Falsey et al., 1995]. In this group most RSV infections are reinfections, which are usually associated with a lower efficacy of virus detection by Ag-ELISA and virus isolation [Falsey et al., 1995].

Encouraging results using reverse transcription (RT) PCR for the detection of RSV in NPAs have been published recently [Paton et al., 1992; Van Milaan et al., 1994; Freymuth et al., 1995]. The aim of the present study was therefore to investigate whether detection of RSV in NPAs can be improved by the use of a sensitive seminested RT-PCR, especially with regard to the diagnosis of RSV infections in older children. In addition the standard PCR procedure was improved by a seminested amplification step. Results obtained by RT-PCR assay (single-step and seminested RT-PCR) were compared to the results of virus isolation and antigen detection with ELISA. In addition, RT-PCR results were also evaluated with regard to the amount of virus-specific IgG antibodies already present at the onset of disease, an indication of reinfection in those above one year of age. It was found that RT-PCR assay is significantly more sensitive for the diagnosis of RSV infection than Ag-detection by ELISA and virus isolation, especially in those more than one year of age.

MATERIALS AND METHODS

Patients and Specimens

Nasopharyngeal aspirates (NPAs) from 132 children hospitalized in the winter months of 1988 to 1992 with various forms of acute respiratory tract infection were

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available in sufficient amounts to be tested for RSV by RT-PCR assay. The aspirates were diluted 1:3 in RPMI and screened by tissue culture virus isolation and enzyme-linked immunosorbent assay (ELISA) for the presence of the following viruses: Influenza viruses A and B, parainfluenza viruses 1, 2 and 3, adenoviruses, rhinoviruses, respiratory syncytial virus and enteroviruses. The remaining portion of each specimen was stored at −70°C. Samples that were positive for RSV and samples that were negative for all of the viruses investigated were selected for examination by RT-PCR assay.

The patients were 1 week to 8 years old with 78 below one year of age (median: 5 months) and 54 above (median: 1.5 years). Acute phase serum samples were drawn from 36 children above one year of age on the day of admission (median: 5 days after onset of symptoms, range: 1–15 days).

Detection of RSV in Nasopharyngeal Secretions

Virus isolation in tissue culture. For the recovery of RSV from nasopharyngeal aspirates HeLa cells, strain "Ohio" [Stott and Tyrrell, 1968] were used. Isolates were identified as RSV by indirect immunofluorescence (IF) using a mixture of nucleoprotein-specific monoclonal antibodies (Chemicon Internat. Inc., Temecula, CA) and fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Jackson Immuno Res. Laboratories, West Grove, PA). Assignment of isolates to subgroup A or B was also performed by IF staining using monoclonal antibodies (obtained from C. Orvell, Department of Virology, Karolinska Institute, School of Medicine, Stockholm, Sweden).

Detection of RSV antigen in NPS. This was carried out by ELISA, as described previously (Popow-Kraupp et al.,1986). Briefly, 50 μl of the diluted and sonicated aspirates was added to the wells of U-shaped removastrips (Dynatech, Plochingen FRG), coated with RSV nucleoprotein-specific guinea pig antiserum and incubated overnight at 37°C. After washing, 50 μl per well of a mixture of RSV nucleoprotein-specific monoclonal antibodies (Chemicon International, Inc., Temecula, CA) was added and incubated for 1 hour at 37°C. Species-specific biotinylated sheep antibodies against mouse immunoglobulin (Amersham Int. Plc., Amersham UK) and streptavidin-POD (Boehringer Mannheim, Germany) was then added and incubation was continued for 1 h at 37°C. Fifty μl of substrate (o-phenylenediamine, 1 mg/ml, 0.1% perhydrol) was added to each well. The reaction was stopped after 30 min by the addition of 100 μl of 2 N H_2SO_4 per well and the absorbance at 492 nm was measured.

Detection of RSV-RNA Sequences

Preparation of samples for RT-PCR. Total RNA was extracted from NPAs or RSV-infected Hep 2 cells as described previously [Casas et al., 1995], but with minor modifications. Briefly 0.1 ml NPA or cell culture extract was thawed and supplemented immediately with 20 U of RNAse inhibitor (Boehringer Mannheim; Germany). Four hundred μl of lysis buffer (4 M guanidinium thiocyanate, 25 mM Na-citrat, 0.5% N-lauroylsarcosine, 1 mM dithiothreitol) and 20 μg glycogen (Boehringer Mannheim; Germany) was added and samples were incubated for 15 min at room temperature. Nucleic acid was precipitated by adding 3 volumes of cold (−20°C) 96% ethanol and one tenth volume of 3M sodium acetat to the whole lysis mixture followed by incubation for 30 min at −70°C. After centrifugation (12000 rpm; eppendorf centrifuge 5415 C) for 10 min ethanol was removed and pellets were washed with cold (−20°C) 70% ethanol, dried and resuspended in 10 μl diethyl pyrocarbonate-treated water. The sensitivity of the RT-PCR assay was assessed by titrating virus stocks containing RSV- (A strain Long- or B strain 9320-) infected Hep 2 cells to determine the 50% tissue cuture infective dose ($TCID_{50}$). Virus stock preparations contained 10^5 $TCID_{50}$/ml for RSV subgroup A and 10^3 $TCID_{50}$/ml for subgroup B, respectively.

Primer sequences. The primers for reverse transcription and first-step PCR were chosen from the gene encoding the F1-subunit of the fusion F glycoprotein, amplifying a fragment of 243 bp from subgroup A and B as described by Paton et al.,1992:

5′-TTAACCAGCAAAGTGTTAGA-3′ (=RSV-1)

5′-TTTGTTATAGGCATATCATTG-3′(=RSV-2)

For seminested PCR we used primer RSV-1 and an inner primer 5′-GGTGTAGGTACACCTGCATT-3′ (=RSV-4) derived from published sequences of RSV-F1-gen [Johnson et al.,1988; Elango et al., 1985] amplifying a fragment of 176 bp.

Reverse transcription. For reverse transcription an aliquot (10 μl) of the extracted RNA was added to the reaction mixture yielding a total volume of 22 μl. The mixture consisted of 50 mM Tris-HCl (ph 8.3), 75 mM KCl, 3 mM MgCl2, 200 μM of each of the dNTPs (dATP, dGTP, dCTP, dTTP), 25 pmol of each primer, 20 U RNase inhibitor and 200 U of cloned murine leukemia virus reverse transcriptase (Gibco BRL). The reaction mixtures were incubated at 42°C for 15 min, and then heated at 99°C for 5 min.

Amplification. For the first-step PCR, 10 μl of the cDNA product was amplified in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (ph 8.3), 2 mM MgCl2, 2 U *Taq* polymerase, 200 μM dNTPs (dATP, dGTP, dCTP, dUTP) and 50 pmol of each oligonucleotide primer (RSV-1, RSV-2). Subsequently, seminested PCR was performed by adding 5 μl of the amplicon to 5 μl of sterile water and 40 μl of the reaction mix described above, except for the appropriate oligonucleotide primers which were RSV-1 and RSV-4 for seminested RT-PCR. The thermocycling procedure for the first-step PCR consisted of 40 amplification cycles (denaturation at 94°C for 30s, primer annealing at 47°C for 40s and extension at 72°C for 30s), followed by reextension at 72°C for 10 min. The seminested step included 35 cycles of denaturation at 94°C for 30s, an-

nealing at 52°C for 40s and extension at 72°C for 10 min.

Gel electrophoresis. Positive reactions were identified by ethidium bromide staining of the characteristic 243 bp (176 bp) product after electrophoresis through a 3:1 NuSieve(R) gel.

Preparation of Stock Virus

Hep 2 cells were used for the passage of RSV stocks (Long strain of subgroup A and strain 9230 of subgroup B). RSV-infected Hep 2 cells served as positive controls and were used in experiments for measuring the sensitivity of RT-PCR assay. The number of Hep 2 cells infected with RSV was determined by immunofluorescence-staining. One hundred µl aliquots of these cells were stored at −70°C and used for estimating the effiency of RT-PCR.

Determination of RSV-Specific Antibodies

RSV-specific IgG antibodies in acute-phase serum samples were determined by ELISA using an antigen prepared from RSV- (Long strain-) infected Hep-2 cells according to the method described by Bruckova et al., [1981]. Mock infected cells were treated in the same way and served as control antigen.

Serum samples were tested at a dilution of 1:100 with biotinylated anti-human IgG (Amersham International plc, Amersham, UK) and streptavidin-POD (Boehringer Mannheim, Germany) were used as conjugates.

RSV specific IgG antibody (expressed as arbitrary units (U)) was quantitated by comparing a 100-fold dilution of the test serum to serial 10-fold dilutions of a standard IgG-positive RSV convalescent serum (complement-fixation titer 1:128), defined as containing 10,000 arbitrary units.

In those cases where acute- and convalescent-phase serum samples were available, a twofold increase in antibody level was considered an indication of recent RSV infection.

Statistical Analysis

In order to identify children with previous RSV infections, a cutoff value for RSV-IgG antibody levels in children above one year of age was set as follows: 50 sera from age-matched controls were selected randomly from sera submitted during the summer, when no acute RSV infection was to be expected. RSV-IgG antibodies were quantitated by ELISA. Statistical analysis of the RSV-IgG antibody units by mixed distribution analysis revealed best fit with a proportion of 56% with previous infection and a cutoff value of 64 U. Fisher exact probabilty test and Mann–Whitney U-test were used to test differences in proportions and units, respectively.

RESULTS

Amplification of Viral RNA from Stock Virus

The sensitivity of the RT-PCR assay was determined by amplification of serial tenfold dilutions of tissue culture-grown virus (Long-strain of subgroup A and strain 9320 of subgroup B). Single-step RT-PCR alone detected approximately 10 $TCID_{50}$, while the seminested step increased assay sensitivity 100 fold, detecting 0,1 $TCID_{50}$ of the strains of both subgroups. Calculations based on the number of RSV-infected Hep 2 cells detectable by RT-PCR assay, revealed that single-step PCR was capable of detecting approximately 100 RSV-infected cells, whereas seminested RT-PCR detected 1 RSV infected cell. The specificity of seminested RT-PCR for RSV was assessed by testing stocks of adenovirus 12, parainfluenza virus 3, rhinovirus 38, coxsackie B3, corona E 229, influenza virus A2 “Victoria” and influenza virus B/Singapore. An appropriately sized band was not visible on agarose gels for all other viruses.

TABLE I. Detection of RSV in NPAs by Seminested RT-PCR, Ag-ELISA and Virus Isolation (VI)

	RT-PCR		
	positive	negative	TOTAL
ELISA/VI			
positive	57 (3)*	2	59
negative	25 (9)	48	73
Total	82 (12)	50	132

*Number of results that were positive only by seminested RT-PCR are shown in parenthesis.

Amplification of Viral RNA in Patient Samples

132 samples were tested by viral culture, Ag-ELISA and RT-PCR assay. The results of these assays are shown in Table 1: RSV-RNA was detectable by seminested RT-PCR in 57 samples that were positive by virus isolation and/or ELISA. Forty-eight samples were negative by all 3 methods. Discrepant results were obtained in 27 samples: Amplifiable nucleic acid could be detected in all but 2 of the samples that were positive by Ag-ELISA or virus isolation. The presence of nonspecific inhibitors of PCR DNA amplification was ruled out by spiking experiments in which serial dilutions of stock virus, added to 100 µl of the original specimens and processed as usual, showed no reduction of sensitivity. In 25 cases laboratory confirmation of clinically suspected RSV-infection could only be provided by the RT-PCR assay, 36% (9/25) of which were only positive by seminested RT-PCR. All samples with positive PCR results were confirmed in a second run and contamination controls were consistently negative.

Pairs of acute and convalescent serum samples were available from four patients whose NPAs were positive only by seminested RT-PCR. Table 2 shows OD_{450nm} values and RSV-specific IgG antibody units of these patients (patient no. 1–4); data are also apparent for one patient in whom RSV was detected by Ag-ELISA, virus isolation and seminested RT-PCR (patient no. 5) and for a further patient whose NPA was negative by all three methods (patient no. 6). In all the four cases the specificity of the PCR result was confirmed serologically by an at least twofold increase in RSV-specific

TABLE II. Serum RSV-IgG Antibody Response and Results Obtained by Seminested RT-PCR and Virus Isolation/Ag-ELISA

Pair no.*	Patients' age	VI/Ag-ELISA	RT-PCR	RSV-IgG antibody units (acute/convalescent)	$OD_{450\ nm}$ (acute/convalescent)
				serum dilution 1:100	
1	5 mo	−	+	28/3800	0,177/0,878
2	4 mo	−	+	88/580	0,335/0,690
3	4 mo	−	+	150/700	0.433/0.732
4	12 mo	−	+	180/7500	0.450/0.929
5	4 years	+	+	250/10.000	0.531/1.404
6	7 mo	−	−	55/38	0.269/0.210

*Serum pair no 1 to 4: positive only by seminested RT-PCR; pair no 5: positive by Virus Isolation/Ag-ELISA and seminested RT-PCR; pair no 6: negative by all three methods.

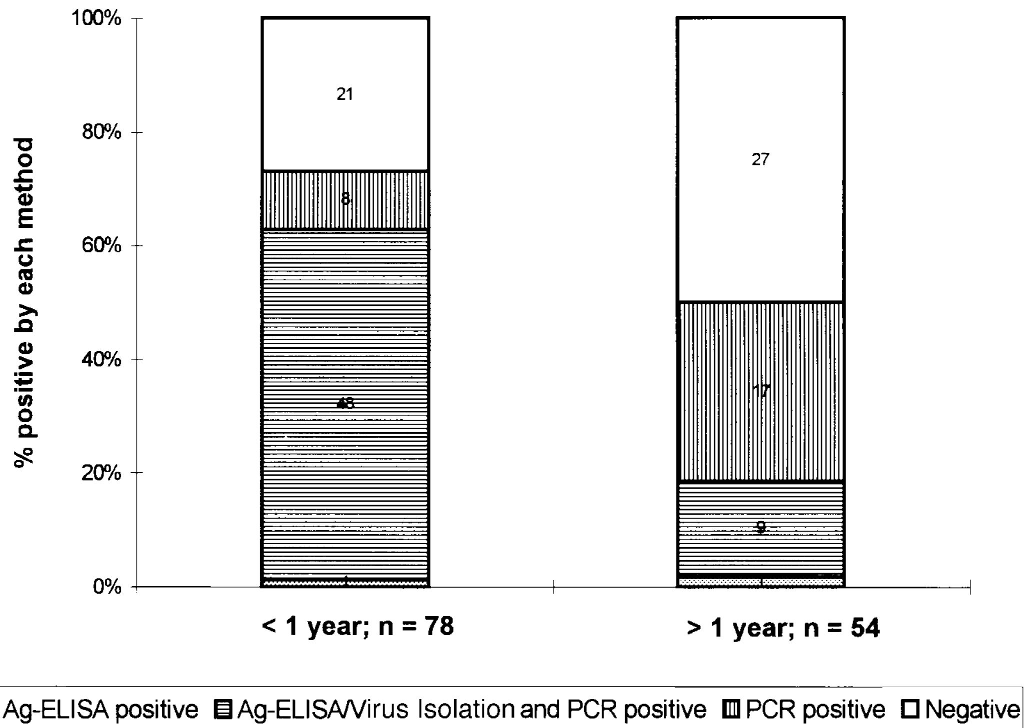

Fig. 1.

IgG antibody units between acute and convalescent phase serum samples, indicating acute infection.

Influence of Patients' Age and Preexisting RSV-IgG on RSV Detection Rate

In order to investigate whether age and previous RSV infection interfere with RSV detection by the methods applied, results obtained by virus isolation, Ag-ELISA and seminested RT-PCR were analysed with regard to the patient's age *(a)* and to RSV-specific IgG antibodies already present at the onset of disease in those above one year of age *(b)*.

a) Patient's age. As shown in Fig. 1, RSV was detected by at least one of the methods applied in 73% (57/78) of the infants less than one year old, compared to 50% (27/54) in those above one year of age. RSV was detected in children below one year of age in 84% (48/57) of RSV-positive samples by all three methods, in 14% (8/57) only by means of RT-PCR assay and in 2% only by ELISA/virus isolation. In contrast, in children above one year RSV was detected in 33% (9/27) of NPAs obtained from RSV infected children by all three methods, in 63% (17/27) only by RT-PCR assay and in 4% (1/27) only by ELISA/virus isolation. The rate of RSV infections detected only by means of RT-PCR assay was significantly higher in children above one year of age, compared to those below ($P < 0.00001$, Fisher's Exact probability test).

Results from the two age groups were compared with regard to the number of days between the onset of symptoms and collection of NPAs to exclude a bias due to differences in stage of illness. The median day of

TABLE III. RSV IgG Antibody Units (U) from 36 Children Above 1 Year of Age and Detection of RSV in NPAs by Seminested RT-PCR, Ag-ELISA and Virus Isolation (VI)

	Ag-ELISA/VI and PCR positive n = 6	Only PCR positive n = 15	Ag-ELISA/VI and PCR negative n = 15
A (IgG < 64 U)	5	1	1
B (IgG < 64 U)	1	14	14
	GMU* = 16[3–82]	GMU = 395[47–2426]	GMU = 765[148–3959]

*Values are given as geometric means with ranges in brackets.

illness when samples were collected was practically the same for both age groups (day 3.96 and day 4.07, respectively).

b)RSV-IgG antibody levels in acute-phase serum samples. To exclude the presence of maternally-derived antibodies, which are usually not detectable beyond the age of one year, this investigation was carried out only with sera available from children above this age. Children were divided into two groups according to the RSV-IgG antibody level (Table 3): Group A (n = 7) included children with a RSV-IgG antibody level <64 U and group B (n = 29) those with a RSV-IgG antibody level >64 U, suggesting previous RSV infections. RSV was detected significantly more often by all three methods (5/6 positive samples) in samples from children of group A, compared to those of group B (1/15 positive samples) ($P = 0.0017$; Fisher Exact probability test), where the majority of RSV infections could only be demonstrated by means of RT-PCR assay (14/15 positive samples). In addition, the geometric means of standard-curve derived arbitrary units of RSV-IgG antibodies (GMU) in patients with a positive PCR, but a negative virus isolation/ELISA result were significantly higher (395; range:47–2426) than that of children with a positive result by all three methods (16; range:3–82) ($P = 0.0031$; Mann–Whitney U-test). The highest RSV-IgG antibody levels (765; range:148–3959) were observed in those children, where RSV infection could not be ascertained by any of the 3 methods applied.

DISCUSSION

We investigated seminested RT-PCR as a method for the rapid diagnosis of RSV infection. Antigen detection by IF or ELISA is a specific, cost-effective and rapid method for RSV diagnosis in young children but is not sensitive enough for the confirmation of clinically suspected RSV infection in older children and in elderly people [Falsey et al., 1995]. Single-step amplification assays for the detection of RSV in NPAs provided a sensitivity equal or slightly superior to those of IF and virus isolation [Cubie et al.,1992; Paton et al.,1992; Van Milaan et al.,1994]. The results obtained by testing virus infected cell lysates showed that the assay sensitivity was increased 100 fold by introducing a seminested step. When this assay was applied to clinical samples, RSV was detected in a significant number of specimens that were negative by ELISA and virus isolation. Since cross-contamination controls were consistently negative and since all positive RT-PCR results could be confirmed in a second run, it is unlikely that the RT-PCR-positive and ELISA/virus isolation-negative samples represent false positive results. In addition, all samples were obtained during winter months with a peak incidence of RSV and were derived from hospitalized infants and children with clinically suspected RSV infection. In children for whom acute and convalescent serum samples were available, positive RT-PCR results were also confirmed by a significant rise in the level of RSV-specific IgG antibodies. Although spiking experiments ruled out a nonspecific inhibition in the two NPAs tested, we cannot exclude that in certain cases inhibitory agents which were subsequently removed by the further dilution procedure in the seminested step may have contributed to the initially negative results obtained by single-step PCR. However, since the majority of single PCR-negative but seminested PCR-positive results were also negative by virus isolation and Ag-ELISA it is more likely that these samples contained RSV in a concentration below the detection limit of single-step PCR, which is about 10 $TCID_{50}$. The majority of the RT-PCR positive and ELISA/virus isolation-negative specimens were obtained from children above one year of age, an age group where 63% of the RSV infections could only be detected by RT-PCR. Results obtained in various studies indicate that the efficiacy of virus detection using ELISA and/or virus isolation is inversely related to the age of the patients [Hornsleth et al.,1982; Falsey et al., 1995]. Large quantities of RSV are usually shed for prolonged periods of time by infants and young children experiencing primary infection. Previous RSV infection does not provide immunity and the virus causes repeated infection and illness throughout life. With an increasing number of subsequent infections, virus is shed in lower quantities for shorter times [Hall et al., 1991] due to the presence of secretory and humoral virus-specific antibodies influencing the degree of viral replication [Mills et al., 1971]. Therefore the significantly higher RSV detection rate with a very sensitive RT-PCR in an age group more likely to experience reinfection is not surprising. This became even more evident when results obtained by ELISA/virus isolation and RT-PCR were analysed with regard to the amount of virus-specific IgG antibodies already present at the onset of disease. Since RSV-specific IgG antibodies detectable in acute phase serum samples are not considered to present the child's ongoing immune response to

the recent RSV infection [Welliver et al., 1980], their presence indicates previous RSV infections in those above one year of age. An inverse correlation between the amount of RSV-specific IgG antibodies and the rate of RSV detection by the three methods was observed. In the majority of children with low or undetectable IgG antibodies RSV was detectable in NPAs by all three methods. In more than 90% of the infants experiencing reinfection, as suggested by the level of preexisting RSV-specific IgG antibodies, RSV was only detectable by means of RT-PCR assay. In children in whom RSV infection could not be proven by any of the three methods applied, significantly higher levels of preexisting virus-specific IgG antibodies were observed than in those with laboratory-confirmed RSV infection. This might indicate that even seminested RT-PCR fails to detect RSV in NPAs of all the reinfected. From the economic point of view, seminested RT-PCR at present cannot compete with the cost- and time-effectiveness of Ag-detection systems. Nevertheless, the significantly higher sensitivity of the RT-PCR for rapid detection of RSV in patients experiencing reinfection, as suggested by the presence of preexisting RSV-specific IgG antibodies, indicates that RT-PCR might be a particularly useful tool for the rapid laboratory confirmation of RSV infection in the elderly, an age group for which currently available diagnostic tests are of little value [Falsey et., 1995].

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